In spite of a common view that aluminum (Al) salts are inert and therefore harmless as vaccine adjuvants or in immunotherapy, the reality is quite different. In the following article we briefly review the literature on Al neurotoxicity and the use of Al salts as vaccine adjuvants and consider not only direct toxic actions on the nervous system, but also the potential impact for triggering autoimmunity. Autoimmune and inflammatory responses affecting the CNS appear to underlie some forms of neurological disease, including developmental disorders. Al has been demonstrated to impact the CNS at every level, including by changing gene expression. These outcomes should raise concerns about the increasing use of Al salts as vaccine adjuvants and for the application as more general immune stimulants.

**Keywords:** adjuvant • aluminum • autoimmunity • CNS • neurodegeneration • toxicity

**Background**

Immune stimulation can occur as the normal response to a foreign pathogen or as an artificial signal designed to stimulate the same immune response. In the latter case, some compounds used in vaccinology, termed 'adjuvants', have been widely used as immune stimulants and have conventionally been considered safe [1]. Of these, the most widely used have been the various salts of Al, used for almost 90 years (since 1926) in a great variety of vaccines [2]. Al salts have also been used in allergy therapy for many decades under the assumption of safety, although convincing data for the latter are still lacking [3]. Al adjuvants act as vehicles for the presentation of antigens not only in the benign sense since they are capable of stimulating pathological immune and inflammatory responses even in the absence of an antigen.

Al is both immuno- and neuro-toxic and in the last decade, studies on animal models and humans have indicated that Al adjuvants have an intrinsic ability to inflict immune and inflammatory responses [4–7]. Notably, the vast majority of adverse manifestations experimentally triggered by Al in animal models, and those associated with administration of adjuvanted vaccines in humans, are neurological or neuropsychiatric [4,6–10]. In this context, recent experiments have revealed that Al adjuvant compounds have a unique capacity to cross the blood–brain and blood–cerebrospinal fluid barriers and incite deleterious immune-inflammatory responses in neural tissues [4,11]. In spite of these data, it is currently maintained by both the pharmaceutical industry and drug regulating agencies that the concentrations at which Al is used in vaccines does not represent a health hazard [12,13]. In the current review we have provided an overview of what is currently known about Al adjuvants, in particular, their modes of action and mechanisms of potential toxicity. We have further addressed the most common misconceptions regarding the safety of Al compounds as adjuvants and the implication of Al’s toxicity in the context of present vaccination and immunotherapy-based medicinal applications.

**Bioavailability of aluminum & its impact on the CNS**

As widely cited, Al is the most common metal and the third most abundant element in the...
Perspective

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earth’s crust [14,15]. In spite of this ubiquity, it has not been widely bioavailable until relatively recent historical periods [16–18] and, perhaps for this reason, seems to have no beneficial role in the biochemistry of any biota [19,20].

The industrial extraction of Al after the early 1800s, primarily from bauxite ore, made it possible to bring Al into a variety of applications from food processing, manufacturing, medicines, dyes, cosmetics, antiperspirants, sun screens and many others [21–23]. One notable addition in recent years has been the widespread use of Al cans and Al foil to store various beverages and food items. Some of these substances are quite acidic and in the absence of adequate or complete coating of the cans, can cause Al to leach into the liquid [24]. Similarly, parenteral nutrition solutions are liable to contamination with Al, particularly from acidic solutions in glass vials, such as calcium gluconate. Because of this, the UK Medicines and Healthcare regulatory Authority (MHRA) recently issued the advice that calcium gluconate in small volume glass containers should not be used for repeated treatment in children <18 years, including in the preparation of parenteral solutions [20]. The advice from the UK MHRA is particularly relevant in light of the findings by Fewtrell et al. who found that parenterally fed preterm infants retain >75% of the Al, with high serum, urine and tissue levels [20]. Notably, the same research group found that preterm infants exposed for >10 days to standard parenteral solutions had impaired neurologic development at 18 months [25]. At 13–15 years, subjects randomized to standard parenterals had lower lumbar spine bone mass; and, in nonrandomized analyses, those with neonatal Al intake above the median had lower hip bone mass [20,26]. Altogether, these studies demonstrated long-term adverse effects of Al on neural development and bone health.

Concerns about the toxicity of ingested Al were expressed over 100 years ago [27], long before it became as widely used as it is today. Nonetheless, it has long been assumed that dietary Al is the main risk source of exposure to biologically available Al. Such false assumptions naturally lead to under-estimation or, even worse, neglect of other sources and routes of Al exposure such as that through skin (i.e., via antiperspirants), nose (via aerosols), as well as medicinal applications (i.e., parenteral feeding solutions and vaccinations) [28,29]. There are thus clearly different routes of Al exposure and what must be emphasized is that these are not necessarily equivalent with regard to the amount of Al delivered per unit of time. For example, although it is commonly assumed that children obtain much more Al from diet than from vaccinations [12], this notion contradicts basic toxicological principles. For instance, the route of exposure that bypasses the protective barriers of the gastrointestinal tract and/or the skin will likely require a lower dose to produce a toxic outcome [28]. In the case of Al, only approximately 0.25% of dietary Al is absorbed into systemic circulation [30]. In contrast, Al hydroxide (the most common adjuvant form) injected intramuscularly may be absorbed at nearly 100% efficiency over time [31].

Similarly to vaccine-derived Al compounds, Al absorbed across the lung or olfactory epithelia by default bypasses the liver and kidney clearance route before encountering the blood–brain barrier. In addition, Al that gains access into the human body through the olfactory system bypasses the defenses of the blood–brain barrier and has direct accesses to the entorhinal cortex and the hippocampal region of the brain, areas which are most vulnerable to neuronal degeneration associated with Alzheimer’s disease [17,28]. Consistent with this observation, abnormally high levels of Al are routinely found in Alzheimer’s brains, with up to fourfold the level of healthy controls. Indeed, sensitive quantifying techniques demonstrate that perikarya of pyramidal cells of the hippocampus and entorhinal cortex are foci where Al accumulation is most pronounced while interneurons are spared [32–34]. Furthermore, several studies have examined the effects of Al on the nervous system function in workers involved in Al production and thus chronically exposed to Al fumes. The findings of such studies suggested a likely role of the inhalation of Al dust in preclinical mild cognitive disorders, which might precede Alzheimer’s disease or Alzheimer’s-like neurological deterioration [35,36].

In regards to dietary Al intake, it is estimated that humans routinely ingest between 2–25 mg per day amounting to 14–175 mg per week [15,37–39]. In urban societies, the intake can exceed 95 mg per day [38], or, 665 mg per week. Because of an increasing consumption of Al-containing convenience foods, in 2006 the Food and Agriculture Organization/WHO Joint Expert Committee on Food Additives [40] amended their provisional tolerable weekly intake for Al from 7 mg per kilogram of bodyweight (amounting to 490 mg per week for an average 70 kg human) to one-seventh of that amount (70 mg per week for a 70 kg human). The Committee concluded that, “Al compounds have the potential to affect the reproductive system and developing nervous system at doses lower than those used in establishing the previous provisional tolerable weekly intake” [40]. Using the estimated intake in urban settings of the higher end of the spectrum of Al consumption referred to above (i.e., 175–665 mg Al per week), it would appear that the ‘average’ consumers weighing 70 kg consume between three- to ten-times the provisionally estimated safe weekly amount of Al according to the standard set by the WHO.
Among the adverse CNS issues in humans linked to Al exposure are: dialysis associated encephalopathy [41,42], autism spectrum disorders [10,43–46] and Alzheimer’s, Parkinson’s disease and related dementias [17,18,47,48], the latter usually seen in aged adults. Al’s toxic effects can manifest as impaired psycho-motor control, altered behavior (i.e., confusion, anxiety, repetitive behaviors, sleep disturbances, deficits of speech, concentration, learning and memory) and seizures [17]. Experiments on cats demonstrated that Al induces neurofibrillary degeneration when present at levels detected in Alzheimer’s patients [49]. This physiological effect was associated with observed impairment in short-term memory and acquisition of a conditioned avoidance response [50].

At a genomic level, Al also causes alterations in DNA transcription. In particular, at nanomolar concentrations, Al inhibits brain-specific gene transcription from selected AT-rich promoters of human neocortical genes [51]. Al’s repressive action on gene transcription is linked to its ability to decrease the access of transcriptional machinery to initiation sites on the DNA template by enhancing chromatin condensation [52,53]; or interfering with ATP-hydrolysis-powered separation of DNA strands either indirectly (by binding to phospho-nucleotides and increasing the stability and melting temperature of DNA [51,54]) or directly (by inhibiting the ATPase-dependent action of RNA polymerase [51]). These effects were experimentally demonstrated at physiologically relevant Al concentrations (10–100 nm [51,55]) and at levels that have been reported in Alzheimer disease patients’ chromatin fractions [56]. It is particularly interesting to note that in spite of its overall repressive action on some gene expression, Al can also promote transcription. Al promotes peroxidation and oxidative stress and in this way activates the reactive oxygen species-sensitive transcription factors, HIF-1 and NF-κB, and augments specific neuroinflammatory and proapoptotic signaling cascades by driving the expression from a subset of HIF-1 and NF-κB-induced promoters [57,58]. Out of eight induced genes upregulated in cultured human neurons by 100 nm Al sulfate (the same compound that is used as a flocculant in water), seven showed expression patterns similar to those observed in Alzheimer’s, including HIF-1/NF-κB-responsive AβPP, IL-1β precursor, NF-κB subunits, cPLA2, COX-2 and DAXX, the latter a regulatory protein known to induce apoptosis and repress transcription [58]. Both HIF-1 and NF-κB are upregulated in Alzheimer’s disease where they fuel the proinflammatory cycle, which leads to further exacerbation of oxidative stress and inflammation, culminating in neuronal death [59].

In light of the above data, we selected 18 candidate genes that are involved in neural functions and innate immune response [60]. In preliminary studies we measured the expression levels of these genes using semiquantitative RT-PCR in brain samples from three CD-1 male controls and three mice injected subcutaneously (s.c.) with Al. The CD-1 mouse model is a good model for toxicity studies as these are heterozygous outbred mice, which is thus representative of the heterozygous human population. In total, seven genes showed changes in expression. Some of the activators and effectors of immunoinflammatory response were significantly upregulated, including IFNG, TNF, chemokine CCL2 and LTB, while the inhibitors of immune reaction NF-κBIB (inhibitor of NF-κB), complement component C2 and a gene controlling the regulation of the degradative enzyme for the neurotransmitter acetylcholine (acetylcholinesterase), were significantly downregulated (Figure 1A & B). In five out of these seven genes, the analysis of the corresponding protein levels showed significant changes in expression: IFNG, TNF and CCL2 were upregulated while NF-κBIB and acetylcholinesterase were downregulated (Figure 1C & D).

Although it is still premature to make definitive conclusions given the relatively small sample size, these results suggest that an immunoinflammatory response was activated and neural activity decreased by Al injection. Moreover, our results are in agreement with Lukiew et al. who demonstrated upregulation of NF-κB responsive and proinflammatory genes by nanomolar Al treatment [58].

Altogether, the gene-expression studies following Al treatment point to a greater complexity than perhaps previously anticipated. Not only can Al evoke direct neural damage and trigger activation of adverse immune-mediated signals, but it can also directly influence gene expression, thus triggering more complex interactions between genes and toxins. Insofar as the latter may be correct, it will be highly important in the future to determine where in the human lifespan can Al impact gene expression and how long such changes might last.

**Key aspects of Al chemistry in relation to biological molecules**

Owing to its 3+ charge, Al attracts negatively charged ions and electrons, but because it cannot transition to other oxidation states besides 3+, Al is not a direct component in any redox reactions, but may participate indirectly in Fenton reactions [61,62].

Moreover, the small ionic radius and the high charge of Al3+ are its important properties by which this metal can exert its toxic activity. The Al ion (0.054 nm) is roughly the same size as the ferric (Fe) ion (0.065 nm) and much smaller than magnesium (Mg; 0.072 nm)
Figure 1. Aluminum administered to mice in vaccine relevant dosages alters the expression of genes involved in immunoinflammatory response and neural function. (A) Aluminum-induced gene-expression alterations in the brains of male CD-1 mice. The expression levels of seven neural and innate immunity-related genes were significantly altered in aluminum-injected compared to control male mice as determined by semiquantitative RT-PCR analyses. β-actin was used as the internal standard. (B) Quantification of the expression change shown in (A). Data are presented as fold difference as compared with controls. Histograms report the mean ± standard error of the mean of three independent experiments determined by densitometry. *p < 0.05; **p < 0.01. (C) The protein levels of the seven genes with altered expression levels after aluminum injection were verified by western blots. β-actin was used as internal standard. (D) Quantification of the protein level change shown in (C). Data are shown as mean signal intensity ± standard error of the mean of three independent experiments. *p < 0.05; **p < 0.01. Al: Aluminum-injected male brains; Con: Saline control males.

and calcium (Ca) ions (0.100 nm). Thus in biological systems, Al can effectively replace these essential biometals in many enzymatic reactions [63–68]. For example, Al binds the extracellular iron carrier transferrin [69–71] which in turn, may facilitate its own transport across the blood–brain barrier [17]. Furthermore, owing to its greater affinity for anionic groups, Al potently interferes with reactions that depend on reversible dissociation. Processes involving rapid Ca$^{2+}$ exchange are inhibited by Al substitution [63,67,68,72]. Similarly, at nanomolar concentrations, Al inhibits many Mg$^{2+}$ and ATP-dependent enzymes, including tubulin GTPase [66] Na$^+$ K$^+$ ATP-ase [73], hexokinase [69,74], RNA polymerase [51,55,75], choline acetyltransferase [76–78], ferrooxidase (ceruloplasmin [79]) and calmodulin-dependent ATPase [65,67,72], as it binds ATP in a complex that is several orders of magnitude more stable than that with Mg (the association constant for Al$^{3+}$ is 10$^7$-times that of Mg$^{2+}$ [66]). Al also binds other nucleotides (GTP and CTP) [80] as well as phosphate headgroups of lipid moi-
eties in membrane systems. Apart from altering membrane properties [81], Al has the potential to interfere with any reaction that requires phosphoryl transfer and ATP/GTP hydrolysis [63,64,72].

Given the ubiquity of enzymatic systems and signaling cascades that depend on G-protein signaling, phosphorylation, ATP, GTP, calcium, magnesium and iron, the spectrum of physiological processes that can be adversely affected by Al is extremely vast. In spite of this, in the absence of chronic renal failure, the toxic effects of Al (especially at low doses) appear to be primarily manifested in the brain [4,15,32,33,69,70,72,81–95], although in vulnerable populations such as infants, prolonged exposure to both high and low doses of Al may also lead to metabolic bone disease [26,96]. Furthermore, Al neurotoxicity appears to be compartmentalized as highly sensitive imaging techniques, as well as methods for quantifying focal accumulations of Al, repeatedly show that Al associates with specific brain regions and cellular compartments, primarily those associated with memory processing and cognitive function [32–34,82,85,97–103].

There are estimated to be 20,000–25,000 protein coding genes in the human genome [104] and even more variant proteins, up to 100,000, that seem to be possible through post-translational modifications. Given this, there are many macromolecules with which Al species can interact. For example, eukaryotic proteins are polypeptides of various combinations and lengths composed of an array of 23 amino acids joined by peptide bonds. Each of the 23 amino acids has a unique side chain consisting of various organic substituents. Al can interact with the side chains [105], some of which — serine, threonine and tyrosine — are phosphorylated, enabling phosphoregulation of enzyme activity and binding with other proteins. Al can disrupt all of these side chains and the processes dependent on them [106].

In summary, for all the above reasons, Al cannot be considered as ‘inert’, nor biologically harmless.

Al, vaccines & vaccine adjuvants

While the bulk of human exposure to Al typically comes from diet, a less obvious but nonetheless not negligible source may be from Al adjuvants used in vaccines. These may include vaccines against diphtheria, tetanus, pertussis, hepatitis B, anthrax, Haemophilus influenzae and human papillomavirus (HPV), among others [12,107,108]. In western countries, a typical child may be injected with as much as 4.225 mg of elemental Al by the age of 12 months [109]. Our review of currently licensed vaccine package inserts in the US is consistent with this figure. For example, according to the standard US vaccination schedule, every vaccinated child will receive a total of 5–6 mg of Al by the age of 2 years, or up to 1.475 mg of Al during a single visit to the pediatrician [17].

Mitkus et al. [109] reported that this dosage is within the US Agency for Toxic Substances and Disease Registry’s minimum risk levels for infants, extrapolating data from a volunteer study of adults using a radioactive Al tracer [110] and a toxicokinetic study performed on rabbits [111]. The authors used the creatinine clearance differential between children and adults to estimate total Al body burden for infants following vaccination [109]. This estimation appears to have been based upon an assumption that Al excretion parallels creatinine clearance, an assumption that is simply incorrect both on theoretical and experimental grounds. Indeed, creatinine clearance in urine is used as a marker for water clearance and it is extremely unlikely that Al excretion follows water. Moreover, rapid excretion of Al would nullify the very basis of having it as an adjuvant in the first place. In particular, although the half-life of enterally or parenterally absorbed Al from the body is short (approximately 24 h), the same cannot be assumed for Al adjuvants as in vaccines. Indeed, experiments in adult rabbits demonstrate that Al hydroxide, the most commonly used adjuvant and immunotherapy Al salt, is poorly excreted. The cumulative amount of Al hydroxide excreted in the urine of adult rabbits as long as 28 days post intramuscular injection was less than 6% as measured by accelerator mass spectrometry [112].

Further research studies show that other than with antigens, Al can form unexpected complexes with other vaccine excipients. Recently, Lee explored the melting profiles of the residual HPV L1 gene DNA contaminant which was detected in the quadrivalent HPV vaccine Gardasil [113]. This quadrivalent vaccine contains genotype-specific L1 capsule proteins of four HPV strains (HPV-16, -18, -6 and -11) in the form of virus-like particles as active ingredients in addition to the Al adjuvant. Because viral DNA fragments if present in the vaccine may bind to the insoluble Al adjuvant (as well as free Al[113]), Lee [113] developed a PCR-based test for HPV L1 gene DNA detection in the final products of Gardasil. The results showed that all samples tested (a total of 16 Gardasil vials) contained residues of the synthetic HPV-11 L1 gene DNA and/or HPV-18 L1 gene DNA. At least seven of the 16 samples also contained HPV-16 L1 gene DNA which was amplified by a pair of modified nondegenerate primers [113]. Notably, the specific melting profile of the HPV-16 L1 gene DNA detected in Gardasil vials was similar to that of the HPV-16 L1 gene DNA recently discovered in the post-mortem blood and spleen of a young woman who suffered a sudden unexpected death 6 months following Gardasil vaccination [114,115]. Collectively, the findings by Lee suggest that the insoluble Al–HPV DNA
complexes may persist in the bodies of vaccine recipients long-term after injection (i.e., up to 6 months), thus perhaps increasing the risk for adverse immune responses [115].

In summary, one of the reasons for the long retention of Al adjuvants in bodily compartments, including systemic circulation, may be due to its tight association with the vaccine antigen or other vaccine excipients (i.e., contaminant DNA). Even dietary Al has been shown to accumulate in the CNS over time, producing Alzheimer’s disease type outcomes in experimental animals fed equivalent amounts of Al to what humans consume through a typical western diet [83,116].

Macrophagic myofasciitis: the Al adjuvant syndrome

The long retention of Al adjuvants was first identified and thereafter extensively studied in macrophagic myofasciitis (MMF) patients. MMF is a condition characterized by highly specific myopathological alterations at deltoid muscle biopsy, first recognized in 1998, and subsequently shown to be due to long-term persistence of vaccine-derived Al compounds within macrophages at the site of previous vaccination – up to 8 to 10 years post injection [6,7,117,118]. Patients diagnosed with MMF tend to be female (70%) and middle-aged at the time of biopsy (median 45 years), and having received one to 17 intramuscular Al-containing vaccine administrations (mean 5.3) in the 10 years before MMF detection [8].

Clinical manifestations in MMF patients include diffuse myalgia, arthralgia, chronic fatigue, muscle weakness and cognitive dysfunction. In particular, up to 93% of patients suffer from chronic fatigue (over 6 months in duration [119]) and up to 89% from chronic diffuse myalgias (over 6 months in duration) with or without arthralgias [8]. Fatigue is disabling in 87% and affects patient’s physical and mental functioning in 53% of cases [119]. Overt cognitive alterations affecting memory and attention are manifested in 51% of cases [8]. In addition to chronic fatigue syndrome, 15–20% of patients with MMF concurrently develop an autoimmune disease, the most frequent being multiple sclerosis-like demyelinating disorders, Hashimoto’s thyroiditis and diffuse immune neuromuscular diseases, such as dermatomyositis, necrotizing autoimmune myopathy, myasthenia gravis and inclusion body myositis [8]. Even in the absence of overt autoimmune disease, low titers of various autoantibodies, increased inflammatory biomarkers and abnormal iron status are commonly detected [120].

The pathological significance of the MMF lesion has long been poorly understood because of the lack of an obvious link between the persistence of Al agglomerates in macrophages at sites of previous vaccination and delayed onset of systemic and neurological manifestations. Nonetheless, that the MMF lesion is linked to a systemic illness was strongly suggested by the fact that a statistically significant association was found between chronic myalgias and fatigue, and the presence of MMF lesions at muscle biopsy in patients. In particular, using electron microscopy, Gherardi et al. [118] detected intracytoplasmic crystalline inclusions typical of the MMF lesion in 40 out of 40 MMF cases and 0 out of 80 controls who suffered from other, MMF-unrelated multisystemic chronic diseases (dermatomyositis or muscle dystrophy). Diffuse myalgias were more frequent in patients with MMF lesions than those without (p < 0.0001).

Medical histories of these cases showed that 50 out of 50 (100%) MMF patients received 1–9 (median 4) doses of Al-containing vaccines within 10 years prior to biopsy. Delay from the last vaccination to biopsy ranged from 3 months to 8 years (median 36 months). Myalgia onset was subsequent to the vaccination (median 11 months) in 94% of patients. Al-containing vaccine administration was carried out prior to onset (44 patients) or worsening (two patients) of myalgias (46 out of 47, 98%). A total of 30% of patients developed myalgias within 3 months after vaccination, 61% within 1 year and 80% within 2 years. A total of 34% of MMF patients also had a concurrent autoimmune disease [118].

Additionally, Gherardi et al. reported the MMF rate of detection in vaccinated patients [118]. In 113 patients with various neuromuscular disorders and previous vaccination with Al-containing vaccines who underwent a deltoid muscle biopsy, 97 (87%) had no detectable MMF lesions, and 16 (13%) had. The delay from immunization to biopsy could be established on the basis of the vaccination booklet in the 16 MMF+ patients and in 81 MMF− patients. The status MMF+ or MMF− could not be attributed to a difference in the delay from immunization to biopsy, this delay being strictly similar in both groups (MMF+ range: 12–96 months, median: 42 months; MMF− range: 3–96, median: 42; MMF+ vs MMF− p = not significant). Out of 16 prospectively detected MMF+ patients, 15 (94%) had typical arthromyalgias and chronic fatigue.

Taken together, these data make a merely coincidental association of MMF with chronic myalgias very unlikely. Moreover, in the series of cases investigated by Gherardi et al., MMF lesions constantly included a lymphoid component ranging from lymphoplasmacytic infiltrates to organized tertiary lymphoid tissue, assessed in an ongoing immunological process at time of biopsy [118]. A persistent systemic immune activation that fails to ‘switch off’ has been regarded as the possi-
able cause of chronic fatigue and arthromyalgias [121,122], through a sustained release of inflammatory cytokines and production of autotoxic T cells and autoantibodies [123–125]. Consistent with this interpretation, Gherardi et al. [118] noted that MMF patients have B-cell hyperlymphocytosis and higher IL-6 circulatory levels than healthy vaccinated controls as well as detectable circulating antinuclear and anti-phospholipid autoantibodies (50%). These data suggest that MMF may be associated with a shift of immune responses towards a Th-2 profile, which is typically induced by Al hydroxide [118], and which probably contributes to the emergence of chronic fatigue and associated manifestations [127].

In summary, these experimental observations cited above demonstrate that not all subjects vaccinated with Al-containing vaccines develop MMF lesions. However, these studies also show that MMF pathology constitutes a systemic illness (with myalgias, arthralgias, chronic fatigue and autoimmune manifestations), rather than a mere local injection-site reaction. Consistent with this hypothesis are the findings of a case–control study on MMF by Bonnefont-Rousselot et al., aimed at determining the presence of oxidative stress in MMF patients [128]. A total of 30 MMF cases (nine males, 21 females; aged 42 ± 14 years), whose diagnosis was confirmed by deltoid biopsy, have been included and compared with 38 sex- and age-matched healthy controls (ten males, 28 females; aged 43 ± 8 years). The blood oxidative stress status was evaluated by assaying six parameters: plasma lipid peroxidation products (thiobarbituric acid-reactive substances) and antioxidant defense systems (plasma vitamin E and glutathione peroxidase (GSH-Px) activity, erythrocyte GSH-Px and SOD activities). The results showed significantly lower levels of plasma GSH-Px activity, selenium and vitamin E concentration in the MMF group compared with the controls (p = 0.004, p = 0.003 and p = 0.009, respectively), with a positive correlation in MMF patients between plasma GSH-Px activity and selenium concentration (p = 0.0001). Given that Al is a well-known pro-oxidant [89], it should not be surprising to find evidence of oxidative stress in MMF. In summary, the case–control studies by Gherardi et al. [118] and Bonnefont-Rousselot et al. [128] both show that MMF constitutes a systemic pathology rather than simply a presence of a benign localized Al-rich muscle lesion as often incorrectly asserted.

**MMF-associated cognitive dysfunction**

As mentioned above, 51% of MMF patients suffer from cognitive alterations [8]. Notably, the MMF-associated cognitive dysfunction (MACD) is a unique MMF-specific phenomenon that provides further evidence for the multisystemic nature of MMF. In particular, unlike other chronic pain syndromes where neuropsychological impairment results from the nonspecific combination of pain, fatigue and depression, MACD seems to reflect a more specific condition, not correlated with pain, fatigue or depression, and independent of symptom duration [6]. This point is of special importance since physicians frequently ascribe cognitive impairment in MMF patients to depression. Thus, although frequently disabling, MACD is often underestimated and underdiagnosed by routine examinations. A comprehensive battery of neuropsychological tests in MMF patients without multiple sclerosis showed alterations in all individuals, consistent with mild cognitive impairment but including at least one test reaching the dementia threshold in 96% of cases [7]. Compared with arthritis controls matched for pain severity and duration, depression and educational level, MMF patients displayed distinctive impairment of visual memory, working memory and dichotic listening, a pattern suggestive of cortico-subcortical organic damage involving frontal-parieto-thalamo-striatal areas, with deep white matter alterations [7].

Although MMF patients do not fulfill the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for dementia, they do present with a notable cognitive complaint. In particular, their neuropsychological evaluation reveals abnormal cognitive performance by age in some domains, consistent with the diagnosis of mild cognitive impairment (MCI) [6]. MCI is a diagnostic entity that was initially set up to identify patients with Alzheimer’s disease at a very early point in the cognitive decline [129]. The term MCI has more generally been used to refer to cognitive dysfunction of insufficient severity to constitute dementia [130]. In applying recent diagnostic scheme for MCI to a cohort of MMF patients, Passeri et al. found that the majority of them fulfilled the criteria for MCI of non- amnestic type, most often of a multidomain nature [6]. However, in most MMF patients, cognitive deficits were sufficiently severe that the term ‘mild’ seemed rather inappropriate, thus leading to introduction of a new MCI subtype, referred to as ‘severe MCI’, in order to characterize patients with lower cognitive deficits [6]. In MMF patients, cognitive dysfunction caused major disability, both in professional skills and daily life [6,7]. This feature is reminiscent of recent observations in very mild Alzheimer’s disease, where the dysexecutive phenotype was associated with more problem solving difficulties than the predominant amnestic phenotype [133].

**Biodistribution of poorly soluble Al-adjuvant nanoparticles across the blood–brain barrier: evidence for understanding the systemic nature of MMF**

Until recently, the cognitive dysfunction in MMF patients has been largely ignored or downplayed by
the medical community despite the fact that MMF remains the most thoroughly investigated post-vaccination condition in which a mechanistic link with Al adjuvants has now been described. In particular, recent experiments in animal models have revealed that a proportion of injected vaccine-derived Al compounds does not stay localized at the site of injection but rather, escapes the muscle mainly within immune cells, thus gaining access to regional lymph nodes. Thereafter, Al-loaded cells exit the lymphatic system, reach the bloodstream (presumably through the thoracic duct) and eventually travel to distant organs including the spleen, liver and the brain, where Al deposits are detected up to 1 year following injection [11]. The neurodelivery of Al adjuvants as well as surrogate compounds (nanoparticle fluorescent surrogates) to the mouse brain was found to be dependent on the monocyte chemoattractant protein 1 (MCP-1/CCL2) as intramuscular injection of murine rCCL2 strongly increased particle incorporation into intact brain while CCL2-deficient mice had decreased neurodelivery.

Regarding the latter finding, the most recent publication by Cadusseau et al. shows that selective elevation of the MCP-1/CCL2 chemokine may represent a biological marker relevant to the pathophysiology of MMF. This outcome again points to a systemic nature of MMF pathology. In particular, Cadusseau et al. performed extensive cytokine screening on the sera from 44 MMF patients and on the sera of sex- and age-matched healthy controls as well as the sera of patients with various types of inflammatory neuromuscular diseases [132]. Thirty cytokines were quantified using a combination of Luminex® technology and ELISA. There was a significant mean increase of serum levels of MCP-1/CCL2 in MMF patients compared with healthy subjects. MMF patients showed no elevation of other cytokines, a result which contrasted with the findings in inflammatory disease patients in whom CCL2/MCP-1 serum levels were unchanged, whereas several other inflammatory cytokines were elevated (IL1β, IL5 and CCL3/MIP1α).

In addition to macrophage-mediated delivery described above, there is a growing body of data to suggest that adjuvant Al is biosequestered by albumin, transferrin and within macrophages of the reticuloendothelial system after intramuscular injection. According to Ganrot [133], insoluble metal hydroxides are thought mainly to be taken up by the reticuloendothelial cells, while soluble salts of trivalent ions are mainly bound to the skeleton or excreted in the urine. Ubiquitous heparan sulfate proteoglycans, which decorate the glyocalyces of our cell membranes, are likely to act as multidentate chelators or biosequestrants of Al [134].

A study on rabbits by Flarend et al. [11] showed that absorption following intramuscular Al particulate injections into the blood was not instantaneous, as only some of the Al was absorbed from the injection depot over the first 28 days. These data are supported by the Khan et al. [11] study suggesting that the initial trajectory for Al hydroxide from the muscle is into the lymphatic system carried by circulating macrophages. Such findings refute the notion that adjuvant nanoparticles remain localized and exert their immunostimulation through a ‘depot effect’. On the contrary, Al from vaccine adjuvants can cross the blood–brain and blood–cerebrospinal fluid barriers and incite immunoinflammatory responses in neural tissues [4,135–137].

These outcomes led Khan et al. to suggest that repeated doses of Al hydroxide are ‘insidiously unsafe’, especially in closely spaced immune challenges presented to an infant or a person with damaged or immature blood–brain or blood–cerebrospinal fluid barriers [11]. Given macrophages acting as highly mobile ‘Trojan horses’ [8], the warning by Khan et al. suggests that cumulative Al from repeated doses in vaccines may produce the cognitive deficits associated with long-term encephalopathies and degenerative dementias in humans [11].

In keeping with the above studies on Al adjuvants and their impact in the CNS, Lujan et al., described a severe neurodegenerative syndrome in commercial sheep linked to the repetitive inoculation with Al-containing vaccines [137]. In particular, the ‘sheep adjuvant syndrome’ mimics in many aspects human neurological diseases linked to Al adjuvants. Moreover, the outcomes in sheep were first identified following a mass-vaccination campaign against blue tongue and have now been successfully reproduced under experimental conditions following administration of Al-containing vaccines. Notably, the adverse chronic phase of this syndrome affects 50–70% of the treated flocks and up to 100% of the animals within a given flock. The condition is made worse by cold weather conditions, suggesting synergy with other stress producing factors. The sheep syndrome is characterized by severe neurobehavioral outcomes – restlessness, compulsive wool biting, generalized weakness, muscle tremors, loss of response to stimuli, ataxia, tetraplegia, stupor, inflammatory lesions in the brain and the presence of Al in the CNS tissues, coma and death [137]. These findings extend those of Khan et al. who demonstrated the ability of Al adjuvants to cross the blood–brain barrier [11], and they further show that Al in the brain can trigger severe long-term neurological damage.

Other animal models show that subcutaneous injections of Al hydroxide induced apoptotic neuronal death
and decreased motor function in mice [4,136]. In newborn mice they were associated with weight increases, behavioral changes and increased anxiety [10].

Cumulatively, the above data may also explain how and why the vast majority of reported adverse reactions following vaccinations are neurological and neuropsychiatric [6–9].

Relation of Al adjuvants to autism spectrum disorders?

Recently, we conducted a study to compare the Centers for Disease Control and Prevention (CDC) recommended vaccine schedules for children’s vaccines in the US (1991–2008) to changes in autism rates during this same period according to data sourced from the US Department of Education (original references in [46]). The data sets, graphed against each other, showed a pronounced and statistically highly significant correlation between the number vaccines with Al and the changes in autism rates. Further data showed that a significant correlation exists between the amounts of Al given to preschool children and the current rates of autism in seven western countries. Those countries with the highest level of Al-adjuvanted vaccines had the highest autism rates. The observed correlation between the number of Al-adjuvanted vaccines and autism was further tested using Hill’s criteria for causality [46] and met eight of nine of these indicating that vaccines containing Al are highly likely to be at least partially causal for autism.

The analyses of the US Vaccine Adverse Events Reporting System (VAERS) database by Seneff et al. likewise appears to support the notion that Al in vaccines is one of the environmental risk factors implicated in autism [45]. In this study, the authors noted that reports of autism in VAERS increased steadily at the end of the last century, during a period when mercury (Hg) was being phased out from vaccines, while the Al adjuvant burden was being increased. Using standard log-likelihood ratio techniques, Seneff et al. have further identified several signs and symptoms that were significantly more prevalent in vaccine reports after the year 2000 (when removal of Hg from vaccines commenced), including cellulitis, seizure, depression, fatigue, pain and death, which are also significantly associated with Al-containing vaccines [45]. That high Al burden might be an etiological factor in autism is further supported by two other recent studies [43,44]. Melendez et al. have shown an elevation of several metals including chromium, arsenic and particularly Al in the blood of autistic children in comparison to the reference values of normal children [43]. Melendez et al. have further identified two important factors regarding exposure to toxic metals: in 80% of cases the autistic children used controlled drugs, and 90% of them had received all recommended vaccines [43]. In addition, 70% of mothers took vaccines and 80% of them ate canned food and fish during pregnancy. Hence the results by Melendez et al. suggest that cumulative exposure to Al from dietary and pharmaceutical sources (i.e., Al-containing drugs and vaccines) in early periods of developmental vulnerability (both pre- and post-natal) may contribute to the development of autism spectrum disorders [45].

Finally, Yasuda and Tsutsui recently summarized the results of a metallomics study in which scalp hair concentrations of 26 trace elements were examined for 1967 autistic children (1553 males and 414 females aged 0–15 years old) [44]. In total, 584 (29.7%), 347 (17.6%) and 114 (5.8%) of children were found to be deficient in zinc (Zn), Mg and Ca, respectively. Both Mg and Ca can be displaced by Al in biochemical reactions as discussed above [17]. In addition, there is data suggesting that Al can also displace Zn [138]. Consistent with these observations, a significant proportion of study children were found to suffer from toxic metal overload, chiefly, Al. In particular, 339 (17.2%), 168 (8.5%) and 94 (4.8%) individuals were found with high burdens of Al, cadmium (Cd) and lead (Pb), respectively, and 2.8% or less from Hg and arsenic (As). Notably, high toxic metal burdens were more frequently observed in infants aged 0–3 years old, whose incidence rates were 20.6%, 12.1%, 7.5%, 3.2% and 2.3% for Al, Cd, Pb, As and Hg, respectively. Yasuda and Tsutsui made an important observation regarding the function of Zn and Zn-finger proteins in transcriptional regulation [44]. Namely Zn-finger proteins influence several candidate genes reported to be associated with the development of autism, such as MTF1, metallothionein, ZnT5, COMMD1, ERK1, TrkB and ProSAP/Shank that themselves are involved in Zn signaling and homeostasis. It is thus plausible that Zn deficiency observed in the autistic subjects might induce critical epigenetic alterations that would further interfere with neuronal maturation during early development.

Altogether, the above findings indicate that Al is yet another environmental agent that can now be added to the list of xenobiotics associated with developmental immunotoxicity (as defined by Dietert and Dietert [139]) and thus an important and yet underappreciated risk factor in disorders of the autism spectrum.

Given all of the above, it appears paradoxical that while there has been a concerted effort to reduce the Al burden in parenteral feedings to premature infants (owing to the observation that 4–5 μg/kg per day of Al can be associated with nervous system and bone toxicity), there has been no concern for the increas-
Immunotherapy reviewed the large body of evidence -47, -...-β-

Although molecular mimicry or a 'bystander activation' of self-reactive lymphocytes, could be the cause for these autoimmune manifestations, the relatively large number and variety of auto-antigens observed (as in the cases of autistic children), points to a polyclonal activation or adjuvant reaction. Moreover, this adjuvant effect, associated with the development of a wide range of autoimmune diseases, has been typically associated with vaccines containing higher levels of adjuvants.

There are several plausible mechanisms that support the role of Al adjuvants in induction of autoimmunity. Particularly notable in this regard is the well-established research on Al's crucial role in activating the NLRP3 inflammasome signaling (and its downstream mediators caspase-1 and IL-1β), which is responsible for the immune adjuvant stimulating properties of Al. Unfortunately, activation of the NLRP3 inflammasome pathway is also critically involved in the development of chronic autoimmune and inflammatory diseases including Type 2 diabetes, demyelinating diseases of the CNS, inflammatory bowel disease, colitis and atherosclerosis. Activation of the inflammasome and its downstream components, proinflammatory cytokines IL-1β and IL-18, is also strongly implicated in promotion of other CNS disorders, including Alzheimer's disease, Parkinson's disease and multiple sclerosis, all of which have independently been linked to environmental Al exposure.

Other vaccine adjuvants may be capable of inducing autoimmune reactions in humans as well. Nohynek et al. [157] and Partinen et al. [158] provided evidence of a significant increase in adolescent narcolepsy in Finland following vaccination with a lipid-based adjuvant in the Pandemrix H1N1 influenza vaccine. These data have now been reproduced in other European countries [159–161]. Whether these outcomes truly reflect negative impacts of the particular adjuvant on the CNS or whether other components of the vaccine alone or in combination with the adjuvant were responsible remains uncertain [159].

Implications for immunotherapy

The demonstrated impact of Al vaccine adjuvants on both the central nervous and immune systems as cited above make it reasonable to question whether the relatively widespread use of Al salts as general immune stimulants in allergy 'immunotherapy' might not also be problematic in the same manner. As recently examined by Exley [3], many of the same considerations apply: Al is neither inert nor harmless in biological systems, it is clearly neurotoxic and can readily enter the CNS by over stimulation of the dog's immune system.

However, the fact that two different vaccines from two different manufacturers were involved strongly suggests a polyclonal activation induced by the vaccine adjuvants without the participation of myelin as the more probable pathogenesis.

Other controlled studies in dogs vaccinated with commercially available rabies and canine distemper vaccines showed a significant increase in the titres of IgG antibodies reactive with ten autoantigens, an effect not observed in unvaccinated dogs [146]. Although molecular mimicry or a 'bystander activation' of self-reactive lymphocytes, could be the cause of the Al adjuvants & ASIA syndrome

Shoenfeld et al. reviewed the large body of evidence which implicates adjuvant administration preceding the onset of immune-mediated diseases including siliconosis, Gulf War syndrome and MMF syndrome. Collectively, these illnesses present similar clinical features which are now designated as being part of a new syndrome called 'autoimmune/autoinflammatory syndrome induced by adjuvants' (ASIA). Many of these appear to arise owing to the use of Al adjuvants [8,144]. Outcomes fitting the ASIA criteria have been reported in sheep following Al adjuvant exposure from vaccines as cited above from the work of Lujan and colleagues [137].

Compelling evidence for a causal role of vaccine adjuvants in triggering serious autoimmune disorders have been presented by Quiroz-Rothe et al. who described a case of post vaccination polyneuropathy resembling Guillain-Barré syndrome in a dog [148]. In this case, there was an apparent cause-effect relationship between vaccination and onset of clinical signs associated with the presence of antibodies against myelin. The authors noted that the vaccines used were obtained by cultures in renal cells and did not contain nervous tissue antigens. Thus either viral or other vaccine antigens, or the adjuvants included in the vaccines, might have triggered the formation of antemyelin antibodies by over stimulation of the dog's immune system.

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also antigenic on its own [165]. Thus, this dual activity raises questions about how the human body reacts to any future exposures to Al [165]. For example, there is evidence that Al in adjuvants is also acting as an antigen as a significant proportion of vaccine recipients retain a ‘memory’ of their exposure to Al, showing delayed hypersensitivity to subsequent exposures to Al [166,167]. Thus, vaccination as well as allergen therapies that incorporate Al-based adjuvants may sensitize recipients to adverse outcomes from future exposures to Al.

Exley et al. further note that the sensitization to Al may simply be one manifestation of the physiological response to biologically available Al [165]. The biological availability of Al, as defined by its propensity to induce a biochemical response in an affected system, is known to depend upon the establishment over time of a threshold concentration, or burden, of Al [168]. The system (i.e., cell or tissue), copes with the burgeoning burden of Al up until a threshold concentration is reached. The immunological memory of early exposures to biologically available Al may vary widely within the recipients such that thereafter there could be many different biochemical responses to a future exposure to Al. In the case of future Al-adjuvant containing vaccinations, the threshold may be achieved instantaneously in individuals who had retained a memory of their earlier exposure to Al and could instigate severe immune responses with wide ranging health implications [165].

The wider cascade of effects may involve the recruitment of Al antigens in other parts of the body or it may be mediated through other antigens that have been sensitized through their previous coadministration with Al adjuvant. An example of this is the sensitization to food allergens following their coadministration with Al salts. Notably, the immunostimulatory properties of Al have been routinely exploited for inducing mast cell-dependent allergic sensitization to food proteins, which ultimately results in intestinal inflammation and diarrhea [169,170]. Mast cells play key roles in a wide range of inflammatory gastrointestinal pathologies in which they compromise mucosal immunity and increase intestinal permeability [169–171]. Particularly relevant in the context of this review is the fact that gastrointestinal dysfunction and food allergies appear to be the most common non-neurological comorbidities in both ASIA and disorders of the autism spectrum [9,171]. These observations provide further compelling evidence supporting the role of Al adjuvant over-exposure in both of these syndromes [43,45,46,141,144].

In summary, an individual susceptibility to an adverse reaction from Al may be dependent upon the combination of a previous sensitization to Al, for example, via childhood vaccination, and an ongoing Al overload from all sources [165]. While the body may copre robustly with a mild but persistent exposure to Al, the coping mechanism will be suddenly and dramatically overwhelmed by a new exposure to Al adjuvant. The latter, will not only enhance the antigenicity itself, but it will raise the level of the immune response against all significant body stores of Al. Under these conditions an individual’s everyday exposure to Al will continue to fuel the response and many symptoms of associated autoimmunity will occur. The individual will now respond adversely to Al exposures, which previously were not sufficient to elicit a biological response [165]. When we take into account that as many as 1% of recipients of Al-containing adjuvants may be sensitized to future exposures to Al [167], then a note of caution could be made regarding future mass overexposure to this form of adjuvant via excessive vaccinations or other forms of immunostimulation (i.e., allergen therapy).

Conclusion

The use of Al salts in vaccines or in immunotherapies may not be as safe as commonly considered owing to Al’s known toxic actions in the nervous system. Furthermore, Al as an antigenic compound can trigger autoimmune reactions. The combination of both actions may render the overuse of Al for such applications ‘insidiously’ unsafe for human health.

Future perspective

Our rapidly growing knowledge of Al actions in the nervous system stands in marked contrast to the increasing use of Al salts for vaccines and general immune stimulation. Based on the current and emerging literature, it seems unlikely that in the future Al will be considered safe for human use in any of the current medicinal applications. If this view is correct, the medical community would be well advised to seek other truly safe adjuvant formulations for vaccines and find other means to stimulate general immune responses.

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Executive summary

- Aluminium (Al) is a known neurotoxin with the demonstrated potential to damage the nervous system in animals and humans.
- Al can also trigger adverse immunoinflammatory reactions.
- The combination of both neuro- and immuno-toxic actions of Al in human medicinal applications (vaccinations and immunotherapy) may render the overuse of Al ‘insidiously’ unsafe for humans.
- Efforts should be pursued by the pharmaceutical industry and regulatory agencies to develop safer adjuvants for human use.

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Are there negative CNS impacts of aluminum adjuvants used in vaccines & immunotherapy? Perspective

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